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Two distinct but interchangeable mechanisms for flipping of lipid-linked oligosaccharides

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Translocation of lipid-linked oligosaccharide (LLO) intermediates across membranes is an essential but poorly understood process in eukaryotic and bacterial glycosylation pathways. Membrane proteins defined as translocases or flippases are implicated to mediate the translocation reaction. The membrane protein Wzx has been proposed to mediate the translocation across the plasma membrane of lipopolysaccharide (LPS) O antigen subunits, which are assembled on an undecaprenyl pyrophosphate lipid carrier. Similarly, PglK (formerly WlaB) is a *Campylobacter jejuni*-encoded ABC-type transporter proposed to mediate the translocation of the undecaprenylpyrophosphate-linked heptasaccharide intermediate involved in the recently identified bacterial N-linked protein glycosylation pathway. A combination of genetic and carbohydrate structural analyses defined and characterized flippase activities in the *C. jejuni* N-linked protein glycosylation and the *Escherichia coli* LPS O antigen biosynthesis. PglK displayed relaxed substrate specificity with respect to the oligosaccharide structure of the LLO intermediate and complemented a *wzx* deficiency in *E. coli* O-antigen biosynthesis. Our experiments provide strong genetic evidence that LLO translocation across membranes can be catalyzed by two distinct proteins that do not share any sequence similarity.

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Introduction

The biosynthesis of N-linked glycoproteins in the endoplasmic reticulum (ER) of eukaryotic cells and in bacteria, as well as the biosynthesis of O-antigen lipopolysaccharide (LPS), exopolysaccharides and murein in bacteria, share two common principles. First, an oligosaccharide intermediate is assembled on an isoprenoid lipid carrier (dolichylpyrophosphate or undecaprenylpyrophosphate, respectively) at the cytoplasmic side of the ER or plasma membrane (Bugg and Brandish, 1994). Second, the lipid-linked oligosaccharide (LLO) is translocated (flipped) across the membrane into the lumen of the ER, the periplasm in Gram-negative bacteria, or the extracellular space in Gram-positive bacteria. Subsequent reactions in these oligosaccharide-dependent pathways differ significantly. The LLO can be modified further as is the case in eukaryotic protein glycosylation (Burda and Aebi, 1999). It can be used as a building block in polymerization reactions (O antigen polysaccharide, capsule, and murein biosynthesis), and may be transferred from the isoprenoid lipid anchor to glycolipid (lipid A-core oligosaccharide) or polypeptide (protein glycosylation) acceptor molecules.

LPS, a major building block of the outer membrane in Gram-negative bacteria, consists of a lipid A-core oligosaccharide and, in some bacteria, an O-specific polysaccharide or O antigen. Two independent pathways are involved in the biosynthesis of the O antigen and the lipid A-core oligosaccharide (Raetz and Whitfield, 2002). O antigens can be homo- or heteropolymers made of oligosaccharide repeats, and they are highly variable within a given species (e.g., about 170 different O serotypes have been identified in *Escherichia coli*) (Orskov *et al*, 1977; Bronner *et al*, 1994). Homopolymeric O antigens are usually synthesized via an 'ABC-transporter-dependent' pathway, where the undecaprenyl-PP-linked polysaccharide is completely polymerized in the cytoplasm and then translocated through an ABC transporter into the periplasm and subsequently transferred to the lipid-A core oligosaccharide by the ligase WaaL (Bronner *et al*, 1994). A '*wzy*-dependent' biosynthetic pathway exists for most heteropolymeric O antigens. In this case, the O antigen subunits are synthesized in the cytoplasm on an undecaprenyl-PP carrier and translocated across the plasma membrane. The O antigen subunits are subsequently polymerized by the concerted action of the Wzy protein and the O antigen chain length regulator Wzz, and finally transferred to the lipid A core oligosaccharide by the WaaL ligase (Valvano, 2003) (Figure 1B). Transmembrane proteins, called Wzx, have been postulated to mediate the translocation of undecaprenyl-PP-linked sugar precursors in the *wzy*-dependent pathway (Liu *et al*, 1996; Paulsen *et al*, 1997; Feldman *et al*, 1999; Marolda *et al*, 2004). Wzx proteins can complement each other in the translocation of different O-antigen sugar precursors (Feldman *et al*, 1999; Marolda *et al*, 2004) and they have very similar hydropathy profiles (MacPherson *et al*,

1995). It has recently been suggested that these proteins can recognize the first sugar phosphate bound to the undecaprenyl-P, which in many cases is *N*-acetylglucosamine (GlcNAc) (Marolda *et al*, 2004). However, no homology or conserved residues are found among Wzx proteins and their primary sequences do not reveal any ATP-binding domains (Wang and Reeves, 1998; Marolda *et al*, 1999, 2004). Thus, the lack of identifiable regions in the primary amino-acid sequence of Wzx proteins contrasts with their proposed ability to mediate a rather conserved process.

A pathway similar to the O antigen biosynthesis was described for protein N-glycosylation in the bacterium *Campylobacter jejuni* (Wacker *et al*, 2002; Young *et al*, 2002), a human pathogen causing gastroenteritis. In this pathway, the heptasaccharide (GalNAc-α1,4-GalNAc-α1,4-[Glc-β1,3-]GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-Bac) is first synthesized in the cytoplasm on an undecaprenyl-PP carrier by the sequential activity of glycosyltransferases encoded by the N-glycosylation operon *pgl* (Figure 1A). This LLO is supposedly transferred across the plasma membrane by an ABC-transporter-like protein, encoded by the *wlaB* gene, and finally N-linked to an asparagine (Asn) residue of target proteins by the oligosaccharyltransferase PglB. The role of the individual glycosyltransferases in this pathway has been defined by *in vivo* and *in vitro* studies (Glover *et al*, 2005; Linton *et al*, 2005; Weerapana *et al*, 2005), but the function of WlaB remains hypothetical.

The N-glycosylation pathway of *C. jejuni* is remarkably similar to the eukaryotic N-glycosylation pathway in the ER. In eukaryotes (Figure 1C), the oligosaccharide biosynthesis starts on the cytosolic leaflet of the ER membrane, but the completion of the oligosaccharide and its transfer to selected asparagine residues in target proteins occur in the ER lumen (Hirschberg and Snider, 1987; Schenk *et al*, 2001). Genetic experiments in the yeast *Saccharomyces cerevisiae* (Helenius *et al*, 2002) identified the RFT1 protein as the putative flippase involved in transferring the dolichyl-PP-linked $Man_5GlcNAc_2$ intermediate across the ER membrane. Similarly to Wzx proteins in bacteria, RFT1 proteins are conserved in eukaryotic organisms. They also have multiple predicted transmembrane regions and lack any characteristic feature in their amino-acid sequences, such as ATP-binding domains.

Therefore, non-ABC- and ABC-type transporters are proposed to catalyze the transbilayer movement of LLOs, a reaction that does not occur spontaneously (Hanover and Lennarz, 1978; McCloskey and Troy, 1980; Bishop and Bell, 1985; Rush and Waechter, 1998; Menon, 1995). We have recently discovered that the *C. jejuni* N-glycosylation system can transfer O polysaccharide from undecaprenyl-PP

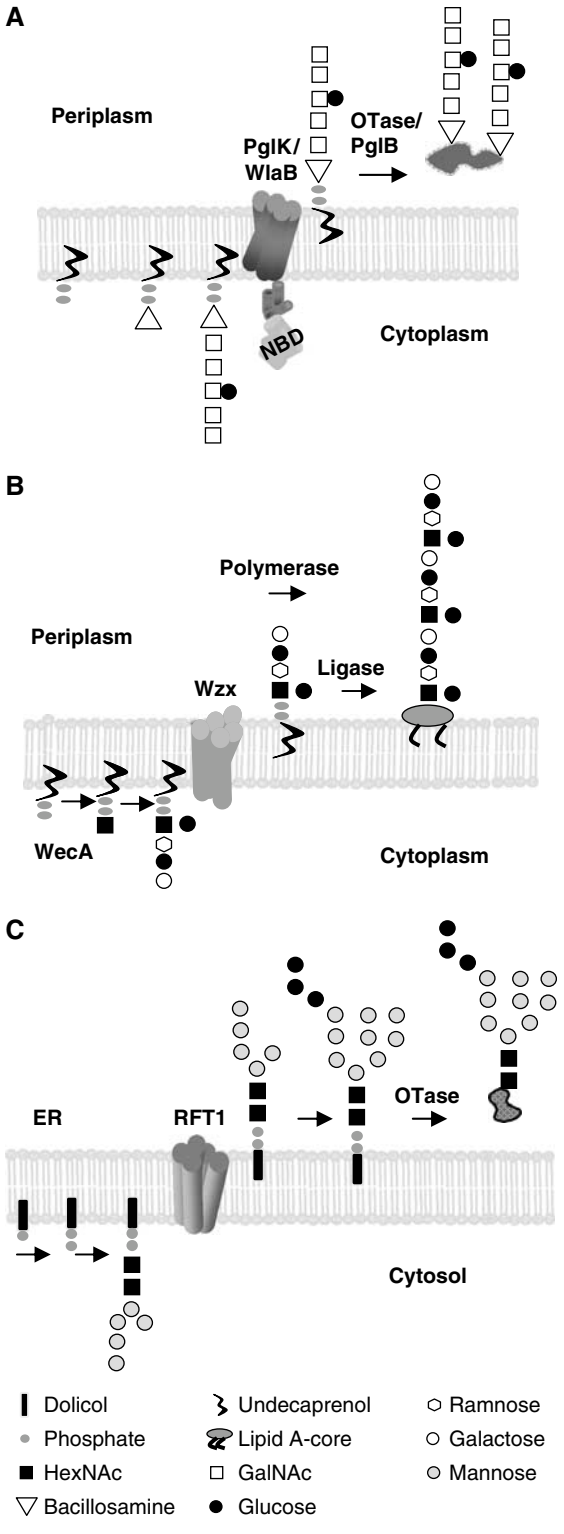

Figure 1 Schematic representation of N-linked protein glycosylation and O-antigen biosynthesis. (**A**) Bacterial N-glycosylation pathway. The oligosaccharide is assembled on the undecaprenyl–P–P carrier by the activity of PglC and additional glycosyltransferases in reactions that occur on the cytoplasmic side of the plasma membrane. The LLO is translocated into the periplasm by the ABC transporter PglK (NBD = nucleotide-binding domain). The oligosaccharyl transferase (OTase) PglB transfers the oligosaccharide to Asn residues of acceptor proteins. (**B**) Wzy-dependent pathway for O-antigen synthesis in Gram-negative bacteria. The O-antigen subunit is assembled onto undecaprenyl–P–P by the activity of WecA and additional glycosyltransferases in reactions that occur on the cytoplasmic side of the plasma membrane. Lipid-linked O-antigen subunits are translocated by Wzx, polymerized and transferred to the lipid A-core oligosaccharide, that is assembled by an independent pathway. (**C**) N-glycosylation in eukaryotes. A dolichylpyrophosphate-linked oligosaccharide is assembled on the cytoplasmic side of the ER membrane and then translocated by RFT1 into the ER lumen. Oligosaccharide synthesis is completed before transfer to Asn residues by the OTase. Symbols have been attributed to the sugar residues according to the CFG nomenclature, the symbols for bacillosamine and ramnose have been newly introduced.

to a periplasmic acceptor protein, a process that requires the activity of the oligosaccharyltransferase PglB (Feldman *et al*, 2005). In this report, we exploited further the commonalities between bacterial N-glycosylation and O-antigen biosynthesis to demonstrate that WlaB is not only responsible for the translocation of the undecaprenyl-PP-linked oligosaccharide in the *C. jejuni* N-glycosylation machinery, but also can mediate the translocation of O-antigen subunits. Therefore, we show that two very different proteins, an ABC-type (WlaB) and a non-ABC-type transporter (Wzx), can exhibit interchangeable roles. Given the defined role of WlaB in the transport of LLOs across the plasma membrane, we propose to rename it as PglK.

Results

N-glycosylation profile of a C. jejuni pglK mutant

A kanamycin resistance cassette was inserted into the *pglK* gene of *C. jejuni* strain 81176 (Korlath *et al*, 1985) to investigate the involvement of PglK in N-glycosylation. The effect of the mutation on the N-glycosylation system was assessed by the analysis of the AcrA protein. AcrA carries two N-linked glycans (Wacker *et al*, 2002) that cause a detectable shift in its electrophoretic mobility, and therefore serves as a probe for the functional status of the N-glycosylation system. Figure 2A shows that the AcrA protein in the wild-type (wt) *C. jejuni* strain has an apparent molecular weight of 47.5 kDa (lane 1), which is consistent with the mass of the diglycosylated form of the protein. In contrast, as reported previously (Wacker *et al*, 2002), inactivation of the PglB oligosaccharyltransferase resulted in nonglycosylated AcrA that migrates faster in the gel (Figure 2A, lane 2). In the *pglK* mutant strain, three AcrA-specific bands were detected (Figure 2A, lane 3). These bands correspond to diglycosylated, monoglycosylated, and unglycosylated forms of AcrA.

The hypoglycosylation phenotype of the *pglK* mutant was confirmed by an experiment using the R12 antiserum. We have sown that this serum reacts with multiple *C. jejuni* proteins and has a high preference for the glyco-epitope (Wacker *et al*, 2002). Inactivation of the *pgl*-dependent general glycosylation system in the *pglB* mutant resulted in a different banding pattern as compared with the wt strain, when membrane extracts were reacted with the R12 serum (Figure 2B, lanes 1 and 2). In contrast, the pattern observed in the membrane extract from the *pglK* mutant corresponded to a mixture of bands found in wt and the oligosaccharyltransferase-deficient cells (Figure 2B, lane 3). In particular, the R12 antiserum detected diglycosylated AcrA in the wt *C. jejuni* (Figure 2B, lane 1) and, confirming the result obtained with the anti-AcrA antiserum, mono- and diglycosylated AcrA in the *pglK* mutant (Figure 2B, lane 3). As expected, AcrA was not glycosylated in the *pglB* mutant (Figure 2B, lane 2).

We concluded from these experiments that inactivation of the *pglK* gene resulted in a hypoglycosylation phenotype, confirming that *pglK* encodes a protein involved in the N-glycosylation process. Given the homology of PglK with ABC-type transporters, it is likely that this protein is involved in the membrane translocation of the LLO intermediate. As the N-glycosylation defect in the *pglK* mutant is only partial, it is possible that other translocases in *C. jejuni* partially complemented the *pglK* defect.

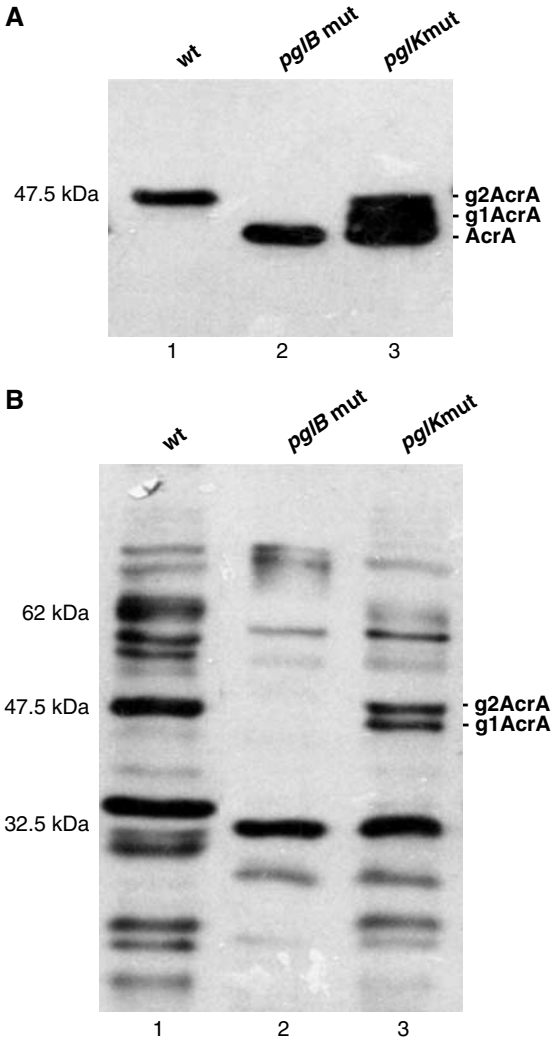

Figure 2 N-glycosylation profile of *C. jejuni pglK* mutant cells. Membrane proteins isolated from *C. jejuni* wt (lane 1), *pglB* (lane 2), and *pglK* (lane 3) mutant cells were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. AcrA and glycosylated proteins were detected with anti-AcrA (**A**) and R12 (**B**) antisera, respectively. The position of bands corresponding to unglycosylated (AcrA), monoglycosylated (g1AcrA), and diglycosylated AcrA (g2AcrA) is indicated.

PglK with a functional nucleotide-binding domain was necessary for N-glycosylation in E. coli SCM7

The *C. jejuni* N-glycosylation pathway can be functionally expressed in *E. coli* (Feldman *et al*, 2005; Wacker *et al*, 2002), making it possible to address the role of individual components of the glycosylation machinery in this heterologous system. To verify the hypothesis that PglK is the LLO translocase of the *C. jejuni* N-glycosylation pathway, it was necessary to construct first an *E. coli* strain lacking all flippases of the Wzx family. This provided a genetic background that prevented potential interference of other LLO flippases present in *E. coli*. For this purpose, we constructed strain SCM7 (see Table I), which has a large deletion eliminating the O antigen and colanic capsule gene clusters and another deletion eliminating the enterobacterial common antigen (ECA) cluster. Therefore, this strain lacks *wzxC* (colanic acid), *wzxO16* (O antigen), and *wzxE* (ECA) flippase genes.

Table I Strains used in this study

Strain	Properties	Source or reference
C. jejuni		
81176	Clinical isolate from a gastroenteritis outbreak	Korlath *et al* (1985)
81176-*pglK*::kan	*pglK* mutant	This work
E. coli		
Top10	F$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcrBC*) f80*lacZ*DM15 Δ*lac*X74 *deoR recA araD* Δ(*ara-leu*) *galU galK rpsL endA nupG*	Invitrogen
SØ874	*lacZ trp* Δ(*sbcB-rfb*) *upp rel rpsL*	Neuhard and Thomassen (1976)
SCM7	SØ874, Δ*wec*	This work
CLM17	W3110, Δ*wzx*	Marolda *et al* (2004)

In order to determine whether PglK can be regarded as an ABC transporter, we investigated the requirement of the ATP-binding domain (NBD), present in the C-terminal part of the protein, for its activity. Conserved amino-acid residues in the Walker A, Walker B and ABC-signature motifs of PglK, some of which are necessary for the ATPase activity of other ABC transporters (Urbatsch *et al*, 2000; Venter *et al*, 2003; Szentpetery *et al*, 2004), were mutated and the glycosylation profile of AcrA in the presence of the mutated PglK forms was analyzed. We also evaluated the function of a PglK mutant carrying the deletion of the entire NBD (ΔNBD) (Venter *et al*, 2003).

Periplasmic protein extracts from *E. coli* strain SCM7 expressing a soluble version of AcrA (Nita-Lazar *et al*, 2004; Feldman *et al*, 2005), the *C. jejuni*-derived *pgl* operon with a deletion of the *pglK* locus, and a wt or mutated version of *pglK* were analyzed by immunoblot using anti-AcrA and R12 antiserum. As noted above, the R12 serum preferentially reacts with the *C. jejuni*-derived glycan.

Unglycosylated AcrA was observed in the absence of functional *PglK* (Figure 3, lane 1), whereas mono- and diglycosylated AcrA proteins were detected in cells where the *pglK* deficiency was complemented by the *pglK* expression plasmid pCA1 (Figure 3, lane 2). AcrA protein was fully glycosylated in the presence of the R492C mutant (Figure 3, lane 8), whereas reduced glycosylation efficiency was observed with mutants L506A and K388A (Figure 3, lanes 4 and 5) and loss of glycosylation was detected in the presence of ΔNBD, S389A, and G488D (Figure 3, lanes 3, 6, and 7). Expression and membrane localization of wt-PglK and the NBD-mutant PglK was confirmed by cell fractionation and subsequent immunodetection with anti-myc antibodies (see Figure S1 in Supplementary data).

The phenotype observed with the *pglK* mutant in *E. coli* SCM7 was identical to that observed with the *pglB* mutant in *C. jejuni* (Figure 2A, lane 2). Thus, these results established our experimental system where PglK activity could be monitored *in vivo* by the analysis of AcrA glycosylation, and suggested that PglK required a functional NBD domain for its function, supporting the notion that it is an ABC-type transporter.

ATPase activity of purified PglK

To verify the ATPase activity of PglK, the protein tagged with a $(His)_{10}$ sequence at the C-terminus was expressed in *E. coli* strain C43 and purified by Ni-NTA affinity chromatography (Catrein *et al*, in preparation). The mutant S389A PglK protein served as a negative control.

Using a colorimetric assay for release of P_i (Chifflet *et al*, 1988), the wt PglK protein showed an ATPase activity of 47 nmol/mg/min (Figure 4A), which is in the range of values reported for other ABC transporters (Schneider and Hunke, 1998). Furthermore, the ATPase activity of wt-PglK was strongly inhibited by addition of orthovanadate (Figure 4B), a well-known inhibitor of ABC transporters (Urbatsch *et al*, 1995a, b). No ATPase activity was detected in the presence of the mutant S389A-PglK.

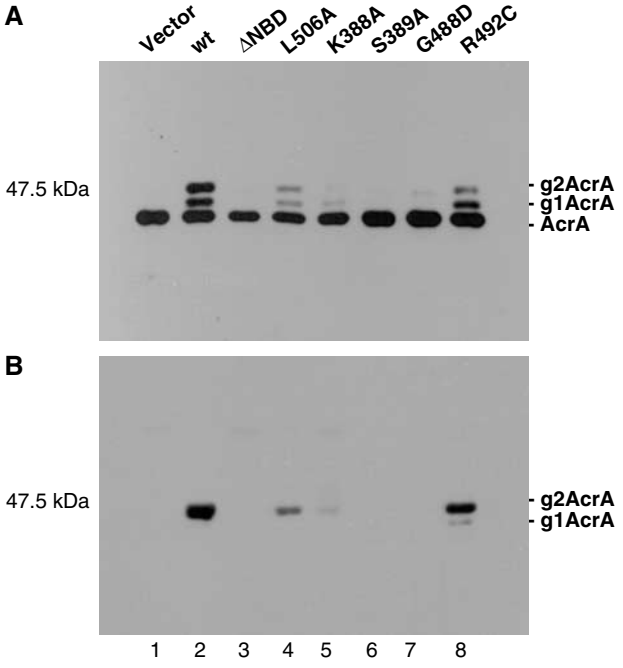

Figure 3 Complementation of a *pglK*-deficient *pgl* operon by PglK and PglK-NBD mutant plasmids. Periplasmic proteins prepared from *E. coli* SCM7 cells carrying the AcrA expression plasmid and the *pglK*-deficient *pgl* operon, complemented with the vector control (lane 1), with a plasmid expressing *pglK*-wt (lane 2) or the *pglK* mutant in the NBD (PglK-ΔNBD in lane 3, PglK-L506A in lane 4, PglK-K388A in lane 5, PglK-S389A in lane 6, PglK-G488D in lane 7, and PglK-R492C in lane 8) were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. AcrA and glycosylated proteins were detected with anti-AcrA (**A**) and the glycoprotein-specific R12 (**B**) antisera, respectively. The position of bands corresponding to unglycosylated (AcrA), monoglycosylated (g1AcrA), and diglycosylated AcrA (g2AcrA) is indicated.

PglK substituted the function of Wzx in O-antigen LPS biosynthesis

To obtain direct genetic evidence for a flippase activity of the PglK protein, we took advantage of the similarity between the N-glycosylation pathway of *Campylobacter* and the LPS

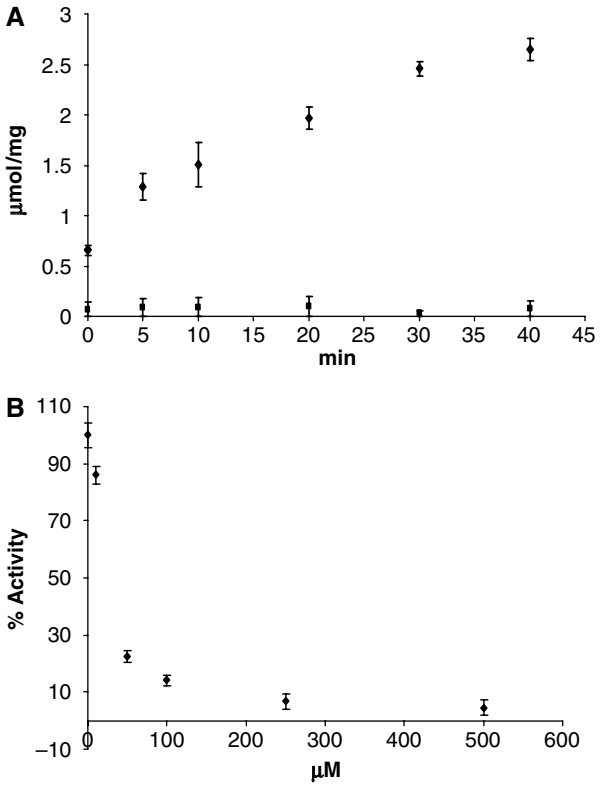

Figure 4 PglK-ATPase assay. (**A**) Purified PglK (◆) and mutant PglK-S389A (■) protein (each 10 µg/ml) was incubated in the presence of 4 mM ATP, 100 mM Tris, pH 7.5, 8 mM $MgCl_2$, 1 mM dithiothreitol, 1% ANAPOE®-$C_{12}E_8$ (Anatrace), 500 mM NaCl, 25 mM Imidazol, and 10% glycerol for various times at 37°C. The generation of P_i was measured at 850 nm. (**B**) The purified PglK protein (◆) (10 µg/ml) was preincubated on ice for 15 min with increasing amounts of sodium orthovanadate (10–500 µM) in the presence of 4 mM ATP, 100 mM Tris, pH 7.5, 8 mM $MgCl_2$, 1 mM dithiothreitol, 1% ANAPOE®-C12E8 (Anatrace), 500 mM NaCl, 25 mM imidazol, and 10% glycerol for 30 min at 37°C, and the generation of P_i was measured at 850 nm. All values are the averages of triplicate determinations shown with standard deviations.

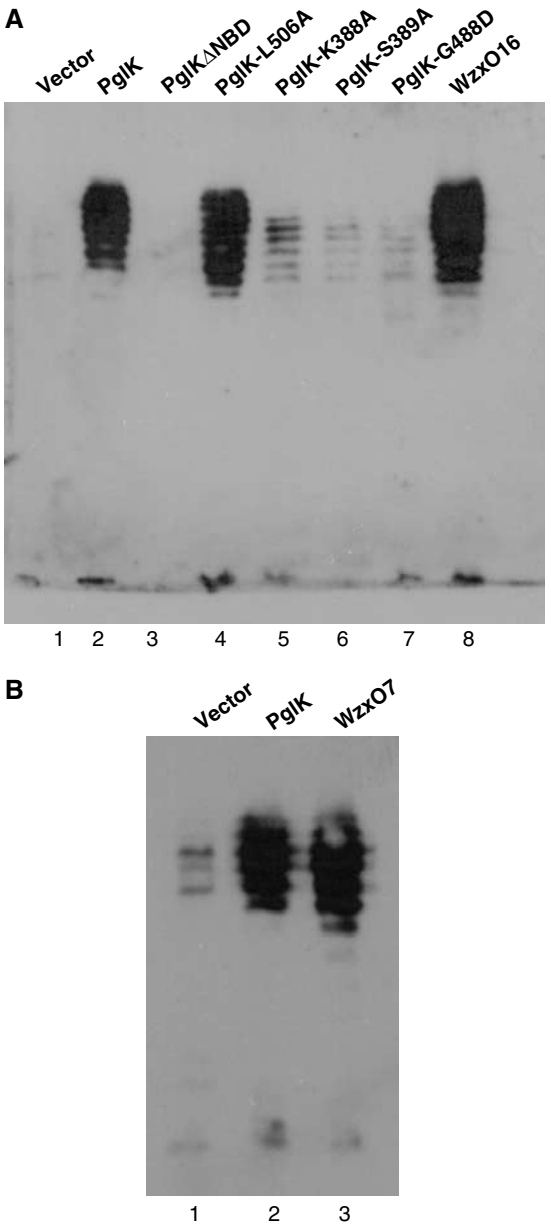

Figure 5 PglK-dependent LPS biosynthesis. (**A**) LPS prepared from *E. coli* CLM17 cells carrying the rhamnosyltransferase-expressing plasmid pMAF19 and a vector control (lane 1), a *pglK*-expressing plasmid (lane 2) with mutations in the NBD (lanes 3–7), or a *wzxO16*-expressing plasmid (lane 8) was separated by 12% SDS–PAGE and transferred to nitrocellulose membranes. O16 LPS was detected with O16-specific antiserum. (**B**) LPS prepared from *E. coli* SØ874 cells carrying cosmid pJHCV32::Tn*3*HoHo1-128, expressing the *wzx* mutant-O7 cluster, with the vector control (lane 1), a plasmid expressing *pglK* (lane 2) or *wzxO7* (lane 3) was separated by 12% SDS–PAGE and transferred to nitrocellulose membranes. O7 LPS was detected with O7-specific antiserum.

biosynthesis pathway of *E. coli*. We investigated whether PglK had the ability to complement O-antigen biosynthesis in the absence of a functional Wzx, the O-antigen translocase. *E. coli* strain CLM17, which carries a *wzxO16* deletion and is defective in O16 LPS synthesis due to *wbbL* mutation (Table I), was used to test PglK-dependent O16 LPS production. CLM17 cells were transformed with plasmid pMF19 (expressing the rhamnosyltransferase *wbbL* gene required for O16 LPS biosynthesis) and with plasmid pCA1 encoding *pglK*. Plasmids pCM223 (encoding WzxO16) and pBAD/Myc-His served as positive and negative controls, respectively. Mutant forms of PglK were also included in this experiment. Biosynthesis of O16 LPS was examined by immunodetection with O16-specific antiserum after LPS separation by sodium-dodecylsulfate (SDS)–PAGE. No O16 antigen was detected in strain CLM17 in the presence of the vector control (Figure 5A, lane 1), whereas formation of O16-specific polysaccharide with the typical ladder-like banding pattern was observed in CLM17 cells expressing WzxO16 (Figure 5A, lane 8) or a functional PglK protein (Figure 5A, lane 2). Thus, we concluded that *pglK* substituted for *wzx* to mediate the translocation of the undecaprenyl-PP-linked O16 subunits. In addition, complementation of the Wzx function required an intact PglK nucleotide-binding domain, since the NBD deletion mutant did not complement (Figure 5A, lane 3), and also the amino-acid replacement mutants in the conserved Walker A and ABC-signature protein motifs provided various degrees of *in vivo* complementation of O16 polysaccharide synthesis (Figure 5A, lanes 4–7).

The ability of PglK to functionally substitute Wzx was investigated further in the O7-LPS biosynthesis pathway. The

O7 and O16 oligosaccharide units have only one hexose, GlcNAc, in common, while the remaining sugars in both subunits are different (L'Vov *et al*, 1984; Stevenson *et al*, 1994) (see Figure S2 in Supplementary data). We introduced into *E. coli* SØ874 the cosmid pJHCV32::Tn*3*HoHo1-128 (Marolda *et al*, 1991, 1999) that carries the O7 antigen synthesis cluster with an inactivated *wzxO7* gene. Either PglK (encoded by plasmid pCW27) or WzxO7 (encoded by plasmid pMF21) complemented O7 polysaccharide synthesis, as demonstrated by immunodetection with O7-specific antiserum, which revealed a typical O antigen ladder in both cases (Figure 5B). This experiment demonstrates that PglK can also complement the *wzxO7* defect. The weak ladder observed in the absence of PglK or WzxO7 (Figure 5B, lane 1) was attributed to partial complementation by the chromosomally encoded WzxE flippase in *E. coli* SØ874.

Altogether, the ability of PglK to complement *wzx* mutations in two different O antigen systems supported its role as a flippase. Moreover, our results indicated a relaxed specificity of PglK activity with respect to the oligosaccharide structure.

The same conclusion, regarding PglK-relaxed specificity, was supported by the analysis of the AcrA-glycosylation phenotype associated to mutant forms of the *pgl* operon (see Figures S3 and S4, in Supplementary data).

Our data supported the hypothesis that PglK is an ATP-dependent LLO flippase with a relaxed specificity with respect to the oligosaccharide structure.

WzxO7 and WzxO16 replaced PglK in the presence of WecA

The interchangeability of the two LLO translocation systems associated with protein glycosylation and O-antigen LPS biosynthesis made it possible to address the substrate specificity of the Wzx flippases. We introduced plasmids encoding PglK, WzxO16, or WzxO7 into *E. coli* SCM7 strain expressing AcrA and the wt or the *pglK*-deficient *pgl* operon. The AcrA-glycosylation profile was analyzed from periplasmic extracts as before. AcrA N-glycosylation was only detected when a functional PglK was expressed (Figure 6, lanes 2 and 5). The inability of the Wzx proteins to complement PglK deficiency suggested that the Wzx-dependent flipping mechanism was not compatible with the components of the N-glycosylation process.

Previous work (Feldman *et al*, 1999; Marolda *et al*, 2004) suggested that Wzx proteins recognize the proximal sugar bound to undecaprenyl-PP. WecA is the UDP-GlcNAc:Und-P GlcNAc-1-P transferase that initiates the biosynthesis of O7 and O16 antigens (Alexander and Valvano, 1994; Yao and Valvano, 1994). WecA also mediates the first step in the ECA biosynthesis (Barr and Rick, 1987), an outer membrane glycolipid made of trisaccharide repeat units produced in all the Enterobacteriaceae (Meier-Dieter *et al*, 1992) via a Wzy-dependent mechanism (Rick and Silver, 1996) that requires WzxE, the translocase of the undecaprenyl–PP–GlcNAc–ManNAc–Fuc4NAc ECA precursor (Rick *et al*, 2003). Since strain SCM7 lacks the ECA cluster, including the *wecA* gene, it cannot synthesize Und–P–P–GlcNAc. We reasoned that the lack of complementation of the *pglK* deficiency by the Wzx flippases could be due to absence of the WecA activity. Therefore, we repeated the PglK-complementation assay described above, but in the presence of a plasmid encoding

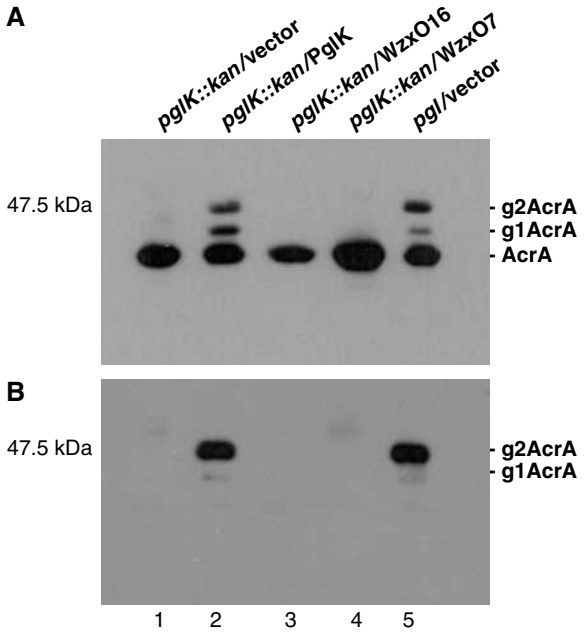

Figure 6 Complementation of PglK deficiency with Wzx proteins. Periplasmic extracts prepared from *E. coli* SCM7 cells carrying the AcrA expression plasmid and the *pgl* operon (lane 5) or the *pglK*-deficient *pgl* operon (lanes 1–4) complemented with the vector control (lanes 1 and 5), a plasmid expressing *pglK* (lane 2), and *wzxO16* (lane 3) or *wzxO7* (lane 4) were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. AcrA and glycosylated proteins were detected with anti-AcrA (**A**) and the glycoprotein-specific R12 (**B**) antisera, respectively. The position of bands corresponding to unglycosylated (AcrA), monoglycosylated (g1AcrA), and diglycosylated AcrA (g2AcrA) is indicated.

WecA. The glycosylation profile of AcrA was analyzed in periplasmic extracts of cells expressing AcrA, the *pglK*-deficient operon, one of the investigated flippases (PglK, WzxO16, WzxO7, or WzxE), and the WecA-expressing plasmid or the vector control. WzxE and WzxO7 complemented the N-glycosylation defect of the *pglK*-deficient operon only when WecA was expressed (Figure 7, lanes 5–6 and 9–10), while PglK activity was independent of WecA expression (Figure 7, lanes 3–4). No significant WzxO16-mediated complementation was observed (Figure 7, lanes 7–8), independently of WecA expression. We concluded that the WzxE and WzxO7 flippases complemented the *pglK* deficiency in a WecA-dependent manner. This is consistent with the observation that, in the presence of a functional WecA and the *C. jejuni pgl* operon, a hybrid *C. jejuni* oligosaccharide initiating with HexNAc instead of Bacillosamine is formed (Wacker *et al*, 2002; Linton *et al*, 2005), and with previous results suggesting that the WzxO7 and WzxE flippases recognize Und–PP–GlcNAc or function in association with WecA (Marolda *et al*, 2004).

Discussion

The translocation of highly hydrophilic LLO across cellular membranes is a key biological reaction in prokaryotes and eukaryotes. However, little is known about the molecular mechanisms of LLO translocation (Menon, 1995). Based on the genetic analysis of LPS biosynthetic pathways in bacteria, it was proposed that translocation can be mediated by

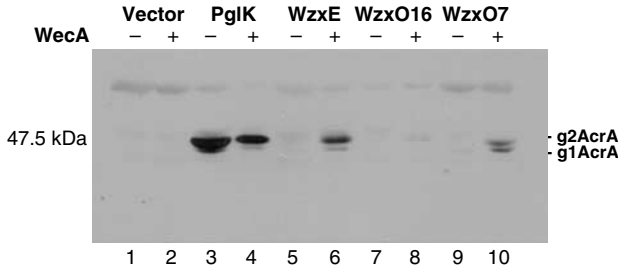

Figure 7 WecA dependency of Wzx proteins. Periplasmic extracts prepared from *E. coli* SCM7 cells carrying the AcrA expression plasmid with the *pglK*-deficient *pgl* operon complemented with the vector control (lanes 1, 2), a plasmid expressing *pglK* (lanes 3, 4), *wzxE* (lanes 5, 6), *wzxO16* (lanes 7, 8), or *wzxO7* (lanes 9, 10), together with the *wecA* expression plasmid (lanes 2, 4, 6, 8, 10) or the corresponding vector control (lanes 1, 3, 5, 7, 9), were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. Glycosylated AcrA was detected with the glycoprotein-specific R12 antiserum. The position of bands corresponding to unglycosylated (AcrA), monoglycosylated (g1AcrA), and diglycosylated AcrA (g2AcrA) is indicated.

proteins belonging to the Wzx or the ABC-transporter family. Interestingly, the Wzx proteins and the eukaryotic RFT1 proteins, the putative flippase in the eukaryotic N-glycosylation system (Helenius *et al*, 2002), are members of the multidrug/oligosaccharidyl/polysaccharide (MOP) exporter superfamily (Hvorup *et al*, 2003), characterized by 12 α-helical transmembrane domains.

Our analysis of the N-linked protein glycosylation pathway of *C. jejuni* revealed that the putative ABC-type transporter protein PglK acts as an LLO flippase in this pathway. We based our conclusion on the experimental observation that PglK activity complemented a *wzx* deficiency and that a PglK deficiency was suppressed by the expression of *wzx* proteins. These results provided direct proof that the flipping of a LLO can be catalyzed by two different types of proteins, most likely by different reaction mechanisms.

PglK activity required a functional ATP-binding cassette (NBD), suggesting that ATP hydrolysis was required to energize the oligosaccharide translocation. Indeed, ATPase activity of PglK was detected in the presence of a wt NBD, and shown to be inhibited by vanadate, a compound commonly used to inhibit ABC transporters. This ATP requirement is in sharp contrast to the *wzx*-mediated translocation, where facilitated diffusion is proposed to be the mechanism underlying the flipping process (Rick *et al*, 2003). Interestingly, the ABC-protein-mediated flipping of LLOs across the membrane usually precedes the final biosynthetic step in different pathways. This is the case for the translocation of the lipid-A core (the final substrate for LPS assembly), the translocation of the LLO in the ABC-transporter-dependent O-antigen biosynthetic pathways, or, as shown in this report, the translocation of the LLO substrate in the *C. jejuni* N-linked protein glycosylation pathway. In contrast, ATP-independent flipping of LLOs is observed in pathways where multiple biosynthetic steps follow the translocation event as in the *wzx*-mediated flipping of *wzy*-dependent O antigens or N-linked protein glycosylation in eukaryotes. Thus, directionality of LLO translocation may be driven in the latter case by the biosynthetic steps following translocation (Rick *et al*, 2003), whereas ATP hydrolysis might be responsible for directionality in the case of ABC-type flippases.

Our finding that the function of different types of LLO flippases can be analyzed *in vivo* using a heterologous system offered unique opportunities to study the substrate specificity of the different flippases. We confirmed previously published results that demonstrated a relaxed specificity of the *wzx* flippases (Feldman *et al*, 1999; Marolda *et al*, 2004). The *wzxE* and the *wzxO7* activity for flipping a *C. jejuni*-derived LLO was WecA dependent, suggesting that GlcNAc as the reducing-end hexose was of central importance for substrate recognition. In the case of the *C. jejuni pglK*, oligosaccharide specificity was even more relaxed and our experiments did not reveal a defined requirement. However, we noted that all the LLO substrates tested in our *in vivo* complementation assay contained a 2-acetamido group in the sugar directly linked to undecaprenylpyrophosphate.

The ability to translocate a wide variety of LLOs across the membrane is therefore a common feature of most of the flippases tested in this report. From a biophysical point of view, the major function of these flippases is the translocation of the hydrophilic oligosaccharide across the membrane and aqueous channels to accommodate theses structures seem the most likely route to perform this activity. However, the presence of such channels would predict a high specificity to prevent translocation of other hydrophilic components across the membrane. It is evident that a detailed biochemical and structural analysis of LLO flippases, as reported for the highly specific flippase MsbA (Doerrler *et al*, 2004a; Reyes and Chang, 2005), is required to establish the translocation mechanism mediated by these enzymes. The fact that two very different types of proteins can catalyze this translocation shows that two distinct mechanisms exist for a reaction that interests both biochemists and biophysicists.

Materials and methods

Bacterial strains, growth conditions, and plasmids

C. jejuni strains were grown on Mueller Hinton agar (Difco) at 42°C under microaerophilic conditions (85% N_2, 10% CO_2, and 5% O_2). *E. coli* strains were grown on Luria Bertani medium at 37°C. Chloramphenicol (20 μg/ml), ampicillin (100 μg/ml), tetracycline (20 μg/ml), spectinomycin (80 μg/ml), and kanamycin (50 μg/ml) were added to the media as needed. Bacterial strains and plasmids used are listed in Tables I and II, respectively.

Construction of a C. jejuni pglK mutant

C. jejuni strain 81–176 (Korlath *et al*, 1985) was transformed with 10 μg of *Nco*I-cleaved pACYC*wlaB*::*kan* DNA (Linton *et al*, 2005), carrying the *pgl* operon with a kanamycin resistance gene cassette inserted in *pglK* (*pglK*::*kan*). For this purpose, five *C. jejuni* colonies were selected and transferred to a fresh plate. The linearized plasmid (in a 20-μl volume) was added on the top of the bacterial colonies, mixed gently and let dry on the plate before incubating for 8 h under microaerophilic conditions at 42°C. Transformants were repurified on plates containing kanamycin. The integration of the transforming plasmid DNA in *pglK* by a double crossover event was confirmed by colony-PCR analysis using primers B3 and B4, annealing to *pglK*, and primers U1 and UL, annealing to *galE* and the kanamycin resistance cassette, respectively. The primers were designed according to the sequenced genome data from the Sanger Center (http://www.sanger.ac.uk/Projects/C_jejuni) (see Table I in Supplementary data). PCR reactions (50 μl) were carried out in 200 mM Tris–HCl, pH 8.8, 20 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1% Triton X-100, 1 mg/ml BSA with 1 μM concentrations of forward and reverse oligonucleotide primers, and 25 U PfuTurbo DNA polymerase (Stratagene). Amplification was achieved with the following thermal cycling conditions: 1 cycle at 95°C for 5 min, 30 cycles consisting of a 95°C denaturation step for

Table II Plasmids used in this study

Plasmids	Description	Source
pACYCpgl	*C. jejuni* pgl cluster, CmR	Wacker *et al* (2002)
pACYC*pglC*::*kan*	*C. jejuni* pgl cluster containing a kan cassette in *pglC*, CmR, KanR	Linton *et al* (2005)
pACYC*pglD*::*kan*	*C. jejuni* pgl cluster containing a kan cassette in *pglD*, CmR, KanR	Linton *et al* (2005)
pACYC*pglE*::*kan*	*C. jejuni* pgl cluster containing a kan cassette in *pglE*, CmR, KanR	Linton *et al* (2005)
pACYC*pglF*::*kan*	*C. jejuni* pgl cluster containing a kan cassette in *pglF*, CmR, KanR	Linton *et al* (2005)
pACYC*pglA*::*kan*	*C. jejuni* pgl cluster containing a kan cassette in *pglA*, CmR, KanR	Linton *et al* (2005)
pACYC*wlaB*::*kan*	*C. jejuni* pgl cluster containing a kan cassette in *wlaB*, CmR, KanR	Linton *et al* (2005)
pACYC*pglH*::*kan*	*C. jejuni* pgl cluster containing a kan cassette in *pglH*, CmR, KanR	Linton *et al* (2005)
pACYC*pglI*::*kan*	*C. jejuni* pgl cluster containing a kan cassette in *pglI*, CmR, KanR	Linton *et al* (2005)
pACYC*pglJ*::*kan*	*C. jejuni* pgl cluster containing a kan cassette in *pglJ*, CmR, KanR	Linton *et al* (2005)
pBAD/Myc-HisA	Cloning vector, AmpR	Invitrogen
pMLBAD	Cloning vector, TpR	Lefebre and Valvano (2002)
pEXT21	Cloning vector, SpcR	Dykxhoorn *et al* (1996)
pBBR1MCS-3	Cloning vector, TetR	Kovach *et al* (1995)
pBBR1MCS-4	Cloning vector,AmpR	Kovach *et al* (1995)
pET22-AcrA	Membrane attached AcrA, AmpR	Linton *et al* (2005)
pCP20	*FLP* + , λ *c*I857 + , λ pR Repts, AmpR, CmR	Datsenko and Wanner (2000)
pKD4	Template plasmid for mutagenesis, AmpR, KanR	Datsenko and Wanner (2000)
pKD46	γ, β, and *exo* from λ phage, *araC-ParaB*, AmpR	Datsenko and Wanner (2000)
pKV1	$wecA_{FLAG/His}$ cloned into pBAD24, Amp^R	K Vigeant
pCM238	*wzxE* cloned into pBAD24, Amp^R	Marolda *et al* (unpublished)
pLMA3	Soluble periplasmic AcrA-(His)6, expression controlled by Tet promoter, in pBBR1MCS-3, TetR	This work
pLMA4	Soluble periplasmic AcrA-(His)6, expression controlled by Tet promoter, in pBBR1MCS-4, AmpR	This work
pCA1	pglK in pBAD/Myc-HisA, AmpR	This work
pCW27	pglK in pMLBAD/Myc-His_6, TpR	This work
pCA1Δ	pglKΔNBD (1–385 aa)	This work
pLM1	pglK-K388A in pBAD/Myc-HisA, AmpR	This work
pLM2	pglK-S389A in pBAD/Myc-HisA, AmpR	This work
pLM3	pglK-L506A in pBAD/Myc-HisA, AmpR	This work
pLM4	pglK-R492C in pBAD/Myc-HisA, AmpR	This work
pLM5	pglK-G488D in pBAD/Myc-HisA, AmpR	This work
pJHCV32::Tn3Ho Ho1-128	O7 LPS biosynthesis gene cluster, wzx::Tn3HoHo1-128, TetR, AmpR	Marolda *et al* (1990)
pMF21	wzx gene of *E. coli* O7 in pEXT21, SpcR	Marolda *et al* (1999)
pCM223	wzx gene of *E. coli* O16 in pBAD24, AmpR	Marolda *et al* (2004)
pMF19	wbbL of *E. coli* O16 in pEXT21, SpcR	Feldman *et al* (1999)

30 s, 50°C annealing for 1 min, and 68°C extension for 2.5 min, and a final extension at 68°C for 10 min.

Construction of E. coli strain SCM7

Deletion of the ECA cluster *wec* in strain SØ874 was performed as described by Datsenko and Wanner (2000). We generated primers *wecA* and *wecG* (see Table I in Supplementary data) of 40–45 nucleotides, corresponding to regions adjacent to the gene targeted for deletion and also containing 20 additional nucleotides that annealed to the template DNA from plasmid pKD4. This plasmid carries a kanamycin-resistance gene flanked by FRT (FLP recognition target) sites. Competent cells were prepared by growing *E. coli* SØ874 carrying pKD46 in LB containing 0.5% (w/v) arabinose and the PCR products were introduced by electroporation. The plasmid pKD46 encodes the Red recombinase of the λ phage, which was placed under the control of the arabinose-inducible promoter P_{BAD}. Kanamycin-resistant colonies were screened by PCR using primers *rho* and *yifK* (see Table I in Supplementary data) annealing to regions outside of the mutated gene. Next, the antibiotic gene was excised by introducing the plasmid pCP20 encoding the FLP recombinase. Plasmids pKD46 and pCP20 are both thermosensitive for replication and they were cured at 42°C.

Construction of recombinant plasmids

E. coli strain Top10 (Invitrogen) was used for DNA cloning experiments and all constructed plasmids were verified by DNA sequencing. All PCR reactions were carried out as described above, using 0.1 μg of template DNA. Plasmid pWA2, expressing a soluble form of AcrA that locates in the periplasm (Feldman *et al*, 2005), was digested with *Ehe*I and *Eco*RI. The resulting 1.7-kb fragment was ligated into plasmid pBBR1MCS-4 (Kovach *et al*, 1995) that had been cleaved with *Sma*I and *Eco*RI, resulting in plasmid pLMA4. A 1.8-kb fragment containing *acrA* under the control of a constitutive promoter was excised from pLMA4 with *Apa*I and *Sac*I and subcloned into *Apa*I–*Sac*I-cleaved pBBR1MCS-3 (Kovach *et al*, 1995), resulting in plasmid pLMA3.

The *pglK* gene was amplified by PCR with oligonucleotides pglK-*Nco*I fw and pglK-*Eco*RI rv. The amplicon was digested with *Eco*RI and *Nco*I and ligated into *Eco*RI–*Nco*I-cleaved pBAD/Myc-HisA (Invitrogen, Carlsbad, CA). This resulted in plasmid pCA1 encoding a PglK with a C-terminal Myc and His_6 tag.

From pCA1, a fragment encoding the *pglK* gene, with the Myc and the His_6 epitope tags, was excised with *Mss*I and ligated into *Sal*I-cleaved pMLBAD plasmid (Lefebre and Valvano, 2002). This resulted in plasmid pCW27.

Plasmid pCA1Δ, expressing a truncated version of PglK (385 amino acids) lacking the nucleotide-binding domain, was constructed in the same way as pCA1, but with ΔNBD-rv as a reverse primer.

The Quick-Change XL Site-Directed Mutagenesis protocol (Stratagene, La Jolla, CA) was followed to introduce the point mutations into the sequence encoding the nucleotide-binding domain of PglK. Primer pairs WA K-A fw and rv, WA S-A fw and rv, ABC G-D fw and rv, ABC R-C fw and rv, and WB L-A fw and rv, together with pCA1 as a DNA template, were used to construct the specific nucleotide substitutions, as suggested by the manufacturer.

A recombinant form of the *wecA* gene was amplified from plasmid pKV1 with primers wecA-Fw and wecA-Rv. The sequence encoding the C-terminal FLAG epitope tag was included in the reverse primer. The 1144-bp amplicon was digested with *Sal*I and *Eco*RI and ligated into *Sal*I–*Eco*RI-cleaved pEXT21 (Dykxhoorn *et al*, 1996), resulting in plasmid pCA21. The *wzxE* gene was amplified from plasmid pCM238 with primers wzxE-Fw and wzxE-Rv. The PCR product was digested with *Eco*RI and *Nco*I and ligated into *Eco*RI–*Nco*I-cleaved pBAD/Myc-HisA, giving rise to plasmid pCE2 encoding WzxE with C-terminal Myc and a His_6 epitope tags. All

plasmids described and primers used are listed in Tables II and I (in Supplementary data), respectively.

Protein expression and immunodetection

Arabinose inducible expression (*pglK*, *pglK* mutants and *wzxE*) was achieved by adding arabinose at a final concentration of 0.2% (w/v) to *E. coli* cells grown in Luria Bertani medium up to an OD_{600} of 0.3. The same amount of arabinose was added again 5 h post-induction, and incubation continued for 15 h. WecA expression was induced by adding IPTG to a final concentration of 0.5 mM when the cultures reached an OD_{600} of 0.3. Induction was maintained for 20 h. The expression of membrane proteins was monitored by fractionation of cells extracts as described previously (Wacker *et al*, 2002). Total *E. coli* cell extracts were prepared for immunodetection analysis using cells at a concentration equivalent to 1 OD_{600} units from overnight cultures, which were resuspended in 50 µl SDS loading buffer (Lemmli, 1970). Aliquots of 10 µl were loaded on 10% SDS–polyacrylamide gels. Periplasmic extracts of *E. coli* cells were prepared by lysozyme treatment (Feldman *et al*, 2005), and 10 µl of the final sample (corresponding to 0.2 OD_{600} units of cells) was analyzed by SDS–polyacrylamide gel electrophoresis.

After being blotted on nitrocellulose membrane (Protran Bioscience, Dassel, Germany, pore size 0.45 µM), the sample was immunostained with the specific antiserum as described (Aebi *et al*, 1996). Anti-AcrA and R12 sera (Wacker *et al*, 2002), anti-Flag (Sigma Aldrich, St Louis, MO) antibodies and anti-Myc (Calbiochem, Darmstadt, Germany) antibodies were used. Antiserum against *E. coli* O7 was obtained from the Statens Serum Institut (Copenhagen). Antiserum against *E. coli* O16 was obtained from the Laboratorio de Referencia de *E. coli* (Lugo, Spain). Anti-rabbit IgG-HRP (Santa Cruz) was used as secondary antibody in combination with the anti-O antigen, anti-AcrA, and R12 antisera. Anti-mouse IgG-HRP (Santa Cruz) was used as secondary antibody in combination with anti-Flag and anti-Myc antibodies. Detection was carried out with ECL™ Western Blotting Detection Reagents (Amersham Biosciences, Little Chalfont Buchinghamshire).

ATPase assay

The wt and S389A mutant PglK protein in 1% ANAPOE®-$C_{12}E_8$ were assayed for ATPase activity at a concentration of 10 µg/ml in 350 µl reaction mixture containing 100 mM Tris–HCl, pH 7.5, 4 mM ATP, 8 mM $MgCl_2$, 25 mM imidazole, 1 mM DTT, 500 mM NaCl, and 10% glycerol. ATPase reactions were incubated for the indicated times at 37°C and 50 µl samples were taken to measure the released P_i (Chifflet *et al*, 1988). If sodium orthovanadate (Sigma) was included in the assay, a 15 min preincubation of all components on ice was performed before the incubation at 37°C for 30 min.

LPS analysis

Cells corresponding to 1 OD_{600} units of an overnight culture were resuspended in 100 µl SDS loading buffer (Lemmli, 1970), heated at 95°C for 5 min, and incubated with 2 µl Proteinase K (50 U/ml; Roche) at 60°C for 2 h. Samples (10 µl) were separated by SDS–PAGE on 12% gels, blotted onto nitrocellulose membranes, and the LPS detected with O7 or O16 specific antiserum as described above.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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